Refinement of Polyclonal Antibody Production by Combining Oral Immunization of Chickens with Harvest of Antibodies from the Egg Yolk

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Abstract

Polyclonal antibody production in mammals is generally associated with multiple injections of antigens and adjuvants and repeated blood sampling procedures. During the past 20 yr, the use of chickens instead of mammals for this purpose has increased. A major advantage of using birds is that the antibodies can be harvested from the egg yolk instead of serum, thus making blood sampling obsolete. In addition, the antibody productivity of an egg-laying hen is much greater than that of a similar sized mammal. This article focuses on the developments in oral immunization strategies for chickens that combined with the antibodies from the egg yolk, have great potential for active implementation of the three Rs (replacing, reducing, and refining the use of laboratory animals to the extent possible) in polyclonal antibody production schemes.

Key Words: adjuvant; chicken; egg yolk; immune system; immunization; oral; polyclonal antibody; three Rs

Introduction

Polyclonal antibodies (PAbs[1]) and monoclonal antibodies (MAbs[1]) are indispensable tools in the laboratory. Recently, laying chickens have attracted considerable attention as an alternative source of antibodies for the prevention and treatment of infectious gastrointestinal diseases. Passive immunization by oral administration of specific egg yolk antibodies against pathogens in both humans and animals is a rapidly growing therapeutic approach for a number of microbial infections (for review, see Mine and Kovacs-Nolan 2002).

We refer readers to the article elsewhere in this issue of *ILAR Journal*, in which Leenaars and Hendriksen (2005) discuss the production of PAbs and MAbs and provide recommendations for effective antibody production while limiting animal welfare problems. Animals used for PAb production often spend months in the animal house, regularly undergoing boosting and bleeding (Hendriksen and Hau 2003). Our objective in this article is to describe alternative procedures for PAb production and to focus on the use of chickens and the exploitation of the egg yolk as a source of antibody in combination with oral immunization. These combined initiatives reduce the potential for distress that is associated with injections and blood sampling procedures.

In most countries, the welfare of laboratory animals used for experimentation is protected by legislation. However, in the case of traditional PAb production, many undesirable features are both necessary and characteristic. For example, the process requires the use of substantial numbers of animals. The experimental procedure is associated with invasiveness, and particularly aggressive immunopotentiating products, termed adjuvants, are used to enhance the immune response. Some routes and locations of the immunization process are considered to be associated with pain and distress. In addition, animals used for production of PAbs may spend a long time in an immunization program. For these reasons, the procedures used to generate PAbs are frequently under animal welfare scrutiny.

Immune Response

The immune response is triggered by contact of the organism with an antigen (von Behring and Kitasato 1890), which is a structure that is recognized by the immune system as foreign ("non-self"). Antigens can be presented to the immune system as complex (particulate) multiantigens (e.g., bacteria, viruses, parasites, and artificial particles) or as single antigens (e.g., proteins or polysaccharides) (Leenaars et al. 1996). An adaptive (antigen-specific) immune system has a high level of specificity and efficiency, but it responds more slowly than the innate unspecific immune system (Hanly et al. 1995). The principal components in the antigen-specific immune response are the following: B lymphocytes, which are responsible for antibody production; T cells (thymus-derived lymphocytes), which are responsible for cytotoxic responses (cytotoxic T cells, T_c cells); and T helper cells (T_h lymphocytes), which are responsible for B cells and T_c cells. Finally, the maintenance of the immune

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[1]Abbreviations used in this article: APC, antigen-presenting cell; BSA, bovine serum albumin; C region, constant region; CT, cholera toxin; CTB, subunit B; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; GI, gastrointestinal; H chain, heavy chain; Ig, immunoglobulin; IgY, chicken egg antibody; IL, interleukin; L chain, light chain; MAb, monoclonal antibody; MHC, major histocompatibility complex; PAb, polyclonal antibody; V region, variable region.

system requires intercellular communication, which is mediated through cell-to-cell contact (e.g., by the production of cytokines) and the support of so-called accessory cells (e.g., fibroblasts and endothelial cells).

The antigen-specific immune response may be divided into three phases, termed the inductive phase, the effector phase, and the establishment of immunological memory (McCullough et al. 1997). In the **inductive phase**, an antigen is recognized as "foreign" by so-called antigen-presenting cells (APCs[1]: monocytes, dendritic cells, and B lymphocytes). Dendritic cells recognize all antigens, whereas B lymphocyte APCs utilize a specific receptor-dependent form of antigen binding. The APC binds and internalizes the antigen and processes it into peptides that, together with major histocompatibility complex (MHC[1]) class molecules, are then exposed on the surface of the APC for presentation to the antigen receptors on the T cells. This contact leads to activation of the T cells. After antigenic contact, only a small percentage of the lymphoid cells become involved in the immune response—initially fewer that 10 per million cells, increasing to one in 1000 cells within 8 days of antigen-dependent activation, when the antigen is a potent immunogen. The remainder of the cells are dormant.

The next step in the immune response is the **effector phase**. Depending on the type of antigen, activation of T cells leads to cell-mediated responses (mainly cytotoxic; e.g., viral antigens) or to T cell help for B cells (e.g., protein antigens). Once activated, T_h cells become more sensitive to the action of growth factor cytokines such as interleukin (IL[1])-1 and IL-2. Cytokines induce the proliferation and ultimately the differentiation of B lymphocytes into antibody-producing plasma cells. Each plasma cell is genetically encoded to produce an exactly defined antibody with specificity against a single antigen epitope only.

Antibodies, also called immunoglobulins or gammaglobulins, are of fundamental importance for the identification and elimination of foreign material that enters the organism. In mammals, antibodies belong to one of five immunoglobulin (Ig[1]) classes—IgA, IgD, IgE, IgG, and IgM—and each antibody has a specific range of activities and characteristics. In avian species, Igs in the circulation belong to one of three classes: IgA, IgM, and IgG. The by far most abundant immunoglobulin in the egg yolk is IgG which is most often referred to as IgY when found in the egg yolk. Avian IgG and IgY are functionally similar to but differ structurally from the mammalian IgG molecule. IgA and IgG may be further subdivided into subclasses in some species. The core structure of all Ig molecules comprises two identical, covalently linked heavy (H[1]) chains and two identical light (L[1]) chains. Each chain has a variable (V[1]) and a constant (C[1]) region. Antibody molecules are bifunctional, having an antigen-binding site and a biological effector function site. The domains of the H and L chain V regions form the antigen-binding sites and differ in structure from one antibody specificity to the next (between clone variation). The H chain C regions (Fc segment) are relatively constant in structure within each subclass of antibodies and contain the biological effector functions such as complement activation.

An antigen epitope consists of approximately five to 10 amino acids. Because most antigens are much larger, they normally constitute a number of different epitopes, each inducing a plasma cell clone (polyclonal) that produces antibodies against a particular epitope. Thus, the antibody response against such antigens normally consists of a combination of a number of monoclonal antibodies (sometimes hundreds to thousands).

The final phase of the antigen-specific immune response is the **induction of memory** after primary contact with the antigen. Memory, which is based on the differentiation of B cells, gives the secondary immune response its characteristics of more speed and greater magnitude than the initial response. In the secondary response, the IgG antibody subclasses are dominant over the IgM response, and the antibodies are of greater avidity compared with the initial response (McCullough et al. 1997).

Scientists who perform one or more immunization procedures typically expect their experimental results to meet numerous conditions and to obtain the following: a high-level antibody titer within a reasonable time; a specific antibody response; induced antibodies that are of high avidity; and an amount of antibody that is sufficient to fulfill the intended result. Generally, many factors involved in an immunization procedure are critical because they may influence both the outcome of the immunization procedure and the welfare of the animals. Leenaars and Hendriksen (2005) provide a detailed description of these factors.

Oral Immunization

Classical immunization for the production of antibodies involves injection of antigen and adjuvants within certain intervals. The preferred routes of administration are subcutaneous and intramuscular (Hendriksen and Hau 2003). However, oral immunization routes are generally considered less stressful for animals, and there has been a welcome development of oral immunization strategies for routine production of antibodies. These routes include oral immunization by voluntary intake of the antigen or by gavage, as well as oral-nasal administration through exposure of the animal to an antigen containing aerosol of the antigen and adjuvants.

The immune system may be viewed as a combination of two systems—the **peripheral** system and the **mucosal** system. Systemic immunity is often a combination of the activation of both systems; however, with respect to the production of specific antibodies against an antigen, it is obviously important to choose an immunization procedure that results in a good response in the peripheral system (i.e., the presence of cellular and humoral immunity in both peripheral and mucosal systems). In addition to antigens that are located predominantly in the lymph nodes, antigens in

blood are filtered, trapped, processed, and presented in strategic blood-tissue interfaces in the spleen, most often resulting in peripheral immunity that is characterized by the appearance of specific IgG. By contrast, antigens in the lumens of enteric organs (i.e., the respiratory and gastrointestinal [GI[1]] tracts) are nondestructively endocytosed by specialized epithelial cells. These antigens are then transcytosed onto lymphoid cells in Peyer's patches, where the response to antigen presentation triggers mucosal immunity that is characterized by release of specific IgA.

Early vaccination studies showed that the route of vaccination was crucial for whether protective mucosal or peripheral immunity would develop. Oral immunization methods for inducing mucosal immunity often delayed or prevented induction of peripheral immunity (MacDonald 1982). The usual response of the GI tract to antigens is tolerance (i.e., no induction of an immune reponse), rather than immunity (Chen et al. 1995); and only certain antigens stimulate a mucosal, peripheral, or combined immune response. The normal healthy GI tract is somehow able to distinguish between safe normal flora and food antigens and dangerous pathogens (Matzinger 1994), but the use of adjuvants and the manipulation of doses can effectively change this. For example, it is easy to hypersensitize mice, rendering them allergic against one or more antigens (e.g., cows' milk proteins) by using very small doses of the antigen (Nielsen et al. 1989; Poulsen et al. 1987).

Oral adjuvants may be classified in the following two groups: those that elicit an immune response themselves, and those whose action relates to antigen uptake of cells. Examples of the latter group include liposomes and nanoparticles. Examples of the first group include cytokines, saponins, and toxin-based adjuvants, one of the most effective and widely used types of adjuvants in oral immunization (Yuki and Kiyono 2003).

One of the enterotoxines used as an adjuvant is cholera toxin (CT[1]), which induces both local and systemic responses (Lycke 1997). Because of the toxicity of CT (Jertborn et al. 1993), only the nontoxic subunit B is used as adjuvant (Jertborn et al. 2001). The subunit B (CTB[1]) is a pentameric structure, which is responsible for the binding of CT to the GM1 receptor present in most cells of the body (George-Chandy et al. 2001; Holmgren et al. 1996; Nashar et al. 1996).

CTB has been demonstrated to be an efficient oral adjuvant (Bergquist et al. 1997; Czerkinsky et al. 1989), but investigators have not yet identified the underlying biological mechanisms with certainty. It has been speculated that the CTB binding to GM1 increases the permeability of the membrane to antigen (Nashar et al. 1996). CTB also induces the MHC class II in B cells and significantly stimulates antigen presentation in macrophages (George-Chandy et al. 2001).

The immune response to an antigen administered with CTB is dependent on whether CTB is conjugated with the antigen or only mixed with an antigen solution (Kang et al. 2003). Although the humoral responses may not differ greatly, a strong cellular immune response appears to require coupling between the antigen and CTB (de Geus et al. 1997; Kang et al. 2003; Tochikubo et al. 1998). Instead of inducing an immune response, CTB coupled to an antigen may in some cases induce immune tolerance to this antigen (Sun et al. 1994). Consequently, CTB administration has been suggested as a possible way to treat autoimmune diseases such as diabetes (Bergerot et al. 1997). CTB has also been demonstrated to be an efficient oral adjuvant in chickens (Hedlund and Hau 2001; Mayo et al. 2003; Takada and Kida, 1996), in which CTB adjuvanticity is correlated with its immunogenicity (Mayo et al. 2005). Also noteworthy is that poly(lactide-co-glycolide) microspheres and dimethyl dioctadecyl ammonium bromide, which have been found to be effective in oral immunization of mammals, were found not to be effective in chickens (Hedlund and Hau 2001).

Chickens and Polyclonal Antibody Production

Because of the continuous transovarian passage of antibodies from blood to egg yolk in birds (Bollen and Hau 1997), it is convenient to harvest and purify antibodies from the egg yolk of the domestic fowl. Several relatively simple methods are described in the literature (Bizhanov et al. 2004; Jensenius et al. 1981; Schade et al. 1996, 2001; Svendsen et al. 1995; Svendsen Bollen et al. 1996). Chicken egg antibodies (IgYs[1]), which are a progenitor of IgG antibodies phylogenetically, can be extracted from the egg to concentrations of approximately 100 mg of IgY/egg. Compared with the IgG productivity of the rabbit (approximately 200 mg of Ig/bleeding), a chicken produces approximately 10 times as much IgY (Bollen and Hau 1996). The concentration of IgY in the growing oocyte from 6 to 38 mm in diameter has been found to be constant. The concentration of immunospecific IgG/IgY has been found to be similar in corresponding serum and yolk samples in chickens immunized with human IgG, and a significant linear correlation between response of a specific antibody in the serum and corresponding egg yolk was found (Bollen and Hau 1997). The serum antibody level must reach a specific level before specific antibody appears in the egg yolk, and to a certain extent, the egg yolk reflects the serum concentration of IgG over 6 to 7 days. This period of time likely corresponds to the time the developing oocyte requires to grow from 6 to 35 mm in diameter (Bollen and Hau 1997).

IgY does not bind to proteins A and G, does not bind mammalian complement (Jensenius et al. 1981), and does not appear to cross-react with mammalian immunoglobulins, thereby reducing the risk for false-positive results in, for example, an enzyme-linked immunosorbent assay (Larsson et al. 1991). Antibody production against highly conserved antigens (e.g., intracellular proteins) requires a wide phylogenetic distance between the recipient and the donor animal (e.g., the use of chickens for producing antibodies to mammalian proteins). Chicken antibodies raised against a

protein in one mammalian species often react against the analogous protein in other mammalian species (Hau et al. 1980, 1981).

The use of chickens for production of antibodies is attractive from an ethical viewpoint and with respect to Russell and Burch's principle of the three Rs (Russell and Burch 1959)—the principle of replacing, reducing, and/or refining the use of laboratory animals when possible. Mammals, which have a higher phylogenetic rank compared with birds, can thus be replaced by a species that has a lower probable degree of neurophysiological sensitivity. In addition, it is possible to reduce the required number of animals considerably when only animals that are similar in body size are compared. The ability to avoid restraining and bleeding animals by substituting chickens for mammals is a considerable element of refinement. Oral immunization techniques are being developed (Mayo et al. 2003; Persdotter Hedlund and Hau 2001) that eliminate restraint and the distress associated with administration of antigen and harvest of antibodies, and that refine the methodology. From a legal perspective, this alternative production of antibodies is no longer considered an experimental procedure.

The use of IgY technology is increasing, and several commercial companies offer an increasing range of different conjugated secondary antibodies. However, the use of mammals still dominates, probably due to a number of factors such as tradition, the infrequent use of the chicken as a laboratory animal in general, and specific housing requirements for the avian species (Leenaars et al. 1999). The fact that IgY must always be purified before use may also be a contributing factor. More information about the IgY technology is available in the literature (Schade et al. 2001).

Immunization schemes that have been developed for other species (e.g., rabbits) normally apply well in the chicken, and most producers of chicken IgY use Freund's adjuvant. Freund's complete adjuvant (FCA[1]) has been associated with severe effects in mammals (Lindblad and Hau 2000). Immunization of chickens using FCA was also found to result in a significant reduction in egg-laying frequency compared with the use of Freund's incomplete adjuvant (FIA[1]) and Hunter's TiterMax in a study by Bollen and Hau (1999b). Although FCA results in higher titers of immunospecific antibody in the egg yolk, this result is counterbalanced by the reduction in the number of eggs produced by the chicken. There is consequently no reason to use FCA in chickens when the egg yolk is used as the antibody source (Bollen and Hau 1999b).

When producing antibodies from chicken eggs, it is common to immunize the chickens when they start laying eggs. Bollen and Hau (1999a) compared the productivity of young chickens immunized at the beginning of the egg-laying period with older chickens immunized during the latter half of the egg-laying period. The older chickens had consistently higher titers than the younger chickens, although the difference was not always significant (Bollen and Hau 1999a).

It has been reported that the physical form of the orally administered antigen is important for the antibody response of chickens: Klipper and colleagues (2001) reported that bovine serum albumin (BSA[1]) in solution was an effective immunogen without adjuvant, whereas BSA powder after administration did not induce an antibody response. When BSA was fed to newly hatched chicks, either in solution or as powder, specific oral tolerance was induced. Ameiss and coworkers (2004) recently reported the interesting finding that ad libitum drinking water administration of a protein antigen was as effective as i.p. administration or gavage routes of antigen administration.

Although oral administration of antigen to newly hatched chickens appears to induce tolerance, it is indeed possible (and may be convenient in large programs) to begin immunization very early. In a recent study by Mayo and coworkers (2003), BSA mixed with a pegylated C8/C10 mono/diglyceride (Rhinovax), or CTB administered orally by gavage to 15-day-old chickens, resulted in circulating immunospecific anti-BSA IgG, IgM, and IgA antibodies. Continuous 5-day oral administration of BSA without adjuvant also resulted in immunospecific IgM and IgA antibodies in the circulation of chickens first immunized at 15 days of age, as well as immunospecific antibodies of all three classes in chickens first immunized when they were 22 days old. IgG and IgM serum concentration levels were, however, more than 4 to 10 times higher, respectively, in CTB- and Rhinovax-treated chickens compared with chickens immunized without adjuvants. The IgA response in the orally immunized chickens appeared unaffected by CTB and Rhinovax. The antibody concentrations in chickens immunized subcutaneously with BSA emulsified in FIA were approximately 10 times higher than those of the chickens orally immunized using CTB and Rhinovax. Although these results may seem disappointing, it is important to remember that the development of efficient oral immunization techniques is still in its infancy. We believe that the combined approaches described above will surely be refined into effective immunization schemes.

Concluding Remarks

Animals used for PAb production often spend months or years subjected to chronic use that involves frequent injections with antigen and adjuvant, restraint, and blood sampling. In the foregoing text, we have focused on the following two approaches to refine production techniques: (1) to replace the mode of administration of antigen and adjuvant from injections to an oral, preferably voluntary, intake of antigen and adjuvant; and (2) to use chickens for antibody production combined with harvest and purification of antibodies from egg yolk, instead of from serum. Combining the two approaches *replaces* the use of mammals with birds, *reduces* the numbers of animals necessary for this purpose, and *refines* the technology with the ultimate goal of eliminating all discomfort for the animals used for this purpose.

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